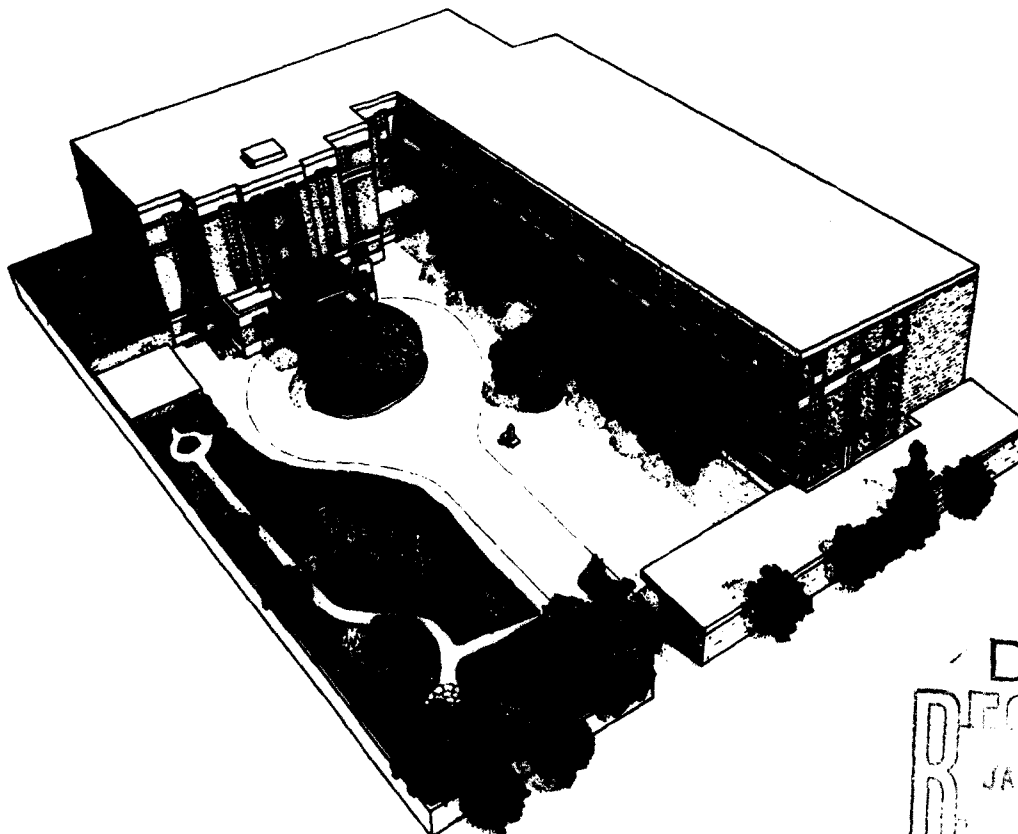


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THE INFLUENCE OF POSTNECROTIC CIRRHOSIS ON
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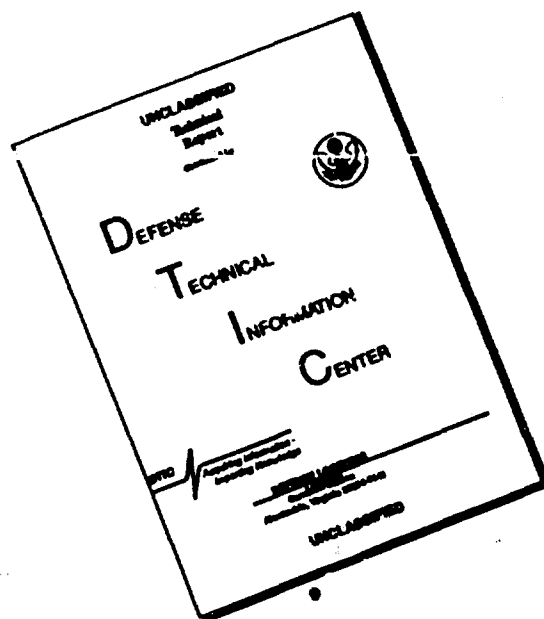
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13. ABSTRACT		
<p>SMALL DOSES OF AFLATOXINS B₁ AND G₁ ADMINISTERED BY INTRAPERITONEAL INJECTION TO RATS WITH CCl₄-INDUCED POSTNECROTIC CIRRHOSIS RAPIDLY CAUSE HEPATOCELLULAR CARCINOMA AND ADVANCED ATYPIA OF LIVER CELLS. FOLLOWING ADMINISTRATION OF 600 G. OF AFLATOXINS IN THREE EQUAL WEEKLY DOSES TO GROUPS OF ANIMALS WITH MODERATE TO SEVERE CIRRHOSIS, 11 OF 16 ANIMALS DEVELOPED HEPATOMA WITHIN 12 WEEKS. WHEN AFLATOXINS OF THE SAME DOSAGE WERE ADMINISTERED TO ANIMALS WITH FATTY LIVERS INDUCED BY ETHANOL, NO HEPATIC TUMOR GROWTHS WERE FOUND. THIRTEEN OF 20 ANIMALS DIED WITHIN 37 WEEKS AFTER ADMINISTRATION OF 1.6 MG. IN EIGHT EQUAL WEEKLY DOSES, INCLUDING THREE WITH HEPATOCARCINOMA AND OTHER WITH PARENCHYMAL LIVER NECROSIS AND/OR PULMONARY INFECTION. AMONG THE SEVEN THAT SURVIVED, TWO DEVELOPED LIVER CELL CANCER AND TWO HAD ATYPICAL DUCTULAR HYPERPLASIA. THE DATA SUGGEST THAT REGENERATIVE LIVER CELLS IN CIRRHOTIC NODULES ARE MORE SUSCEPTIBLE TO ENVIRONMENTAL CARCINOGENS SUCH AS AFLATOXINS.</p>		

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The Influence of Postnecrotic Cirrhosis on Aflatoxin Carcinogenesis in Rats

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Small doses of aflatoxins B₁ and G₁ administered by intraperitoneal injection to rats with CCl₄-induced postnecrotic cirrhosis rapidly cause hepatocellular carcinoma and advanced atypia of liver cells. Following administration of 600 µg. of aflatoxins in three equal weekly doses to groups of animals with moderate to severe cirrhosis, 11 of 16 animals developed hepatoma within 12 weeks. When aflatoxins of the same dosage were administered to animals with fatty livers induced by ethanol, no hepatic tumor growths were found. Thirteen of 20 animals died within 37

weeks after administration of 1.6 mg. in eight equal weekly doses, including three with hepatocarcinoma and others with parenchymal liver necrosis and/or pulmonary infection. Among the seven that survived, two developed liver cell cancer and two had atypical ductular hyperplasia. The data suggest that regenerative liver cells in cirrhotic nodules are more susceptible to environmental carcinogens such as aflatoxins.

Additional key words: Liver cell carcinoma.

Cirrhosis of the liver has long been implicated in the development of primary liver carcinoma.² It has been postulated that regenerative nodules in postnecrotic cirrhosis may be precursors of liver cancer. A recent review considers the influence of aflatoxins, which are food contaminants in some parts of the world, in the causation of hepatoma.¹ On the basis of the supposition that the nodular hyperplasia occurring in postnecrotic cirrhosis is a predisposing factor in the histogenesis of liver cancer, the effect of postnecrotic cirrhosis induced by carbon tetrachloride (CCl₄) on aflatoxin carcinogenesis was studied in rats by using small doses of these toxins and employing a shorter period of time. This paper presents the results of these observations.

MATERIAL AND METHODS

Aflatoxins were produced by *Aspergillus flavus*, ATCC 15517, grown on semisynthetic medium,⁴ extracted with chloroform, and assayed quantitatively and qualitatively by a thin layer chromatographic method as described by Shotwell *et al.*¹² A crude aflatoxin preparation containing B₁, 57 per cent, G₁, 41 per cent, and B₂ and G₂ in trace amounts was used in experiments. The toxins were dissolved in *N,N*-dimethyl formamide-propylene glycol (1/1, v/v), with a total concentration of 1.0 mg. per ml.

Four experiments on aflatoxin carcinogenesis were conducted on albino rats pretreated with carbon tetrachloride and or ethyl alcohol. In each experiment, equal numbers of male and female animals weighing 150 to 200

gm. were kept in separate cages and maintained on a laboratory chow diet.

EXPERIMENT 1

This experiment was designed to study the short term effect of aflatoxins on postnecrotic cirrhosis of moderate degree. The cirrhosis was induced by administration of carbon tetrachloride, 0.2 ml. of a 50 per cent concentration in vegetable oil, twice a week by subcutaneous injection for 12 consecutive weeks. Four randomly selected animals were sacrificed 14 to 16 weeks after the first injection of CCl₄ for histologic confirmation of the degree of cirrhosis. The 20 remaining animals, male and female in equal number, were subjected to further aflatoxin administration after a 4- to 6-week resting interval. The animals received intraperitoneal injections of 200 µg. of aflatoxins in 0.2-ml. prepared solutions once a week for 3 consecutive weeks. The animals were kept for another 9 weeks before postmortem examination.

EXPERIMENT 2

This group of animals was pretreated with carbon tetrachloride and ethyl alcohol to induce severe postnecrotic cirrhosis. The same doses of CCl₄ for the same period of time were administered, and simultaneously 5 per cent ethyl alcohol in water *ad libitum* was substituted for water. After 12 weeks, the 10 animals that survived were subjected to aflatoxin administration. They were allowed normal drinking water and were maintained for another

TABLE 1. PRECANCEROUS AND CANCEROUS LESIONS INDUCED BY AFLATOXINS IN RATS WITH PREDISPOSED POSTNECROTIC CIRRHOSIS

Experimental group	No. of animals (sex)	Pretreatment	Toxin doses	Duration	No. of animals survived	No. with hepatic lesions		
						Advanced atypia	Hepatoma	Atypical ductular hyperplasia
			$\mu\text{g.} \times \text{wk.}$	$\text{wk. after first injection}$				
Postnecrotic cirrhosis, moderate	10 (M)	0.2 ml. CCl_4 of 50% conc. $\times 24$, twice weekly	200×3	12	5	1	4	0
	10 (F)				6	3	3	0
Postnecrotic cirrhosis, severe	5 (M)	0.2 ml. CCl_4 of 50% conc. $\times 24$, twice weekly, plus 5% ethanol <i>ad libitum</i> $\times 12$ wk.	200×3	12	2	0	2	0
	5 (F)				3	1	2	0
Postnecrotic cirrhosis, moderate	10 (M)	0.2 ml. CCl_4 of 50% conc. $\times 24$, twice weekly	200×8	37	2	1	1	0
	10 (F)				5	2	1	2
Fatty metamorphosis	5 (M)	5% ethanol <i>ad libitum</i> $\times 12$ wk.	200×3	12	5	0	0	0
	5 (F)				4	0	0	0
Normal liver	5 (M)	None	200×3	12	5	0	0	0
	5 (F)				5			
Normal liver	5 (M)	None	200×8	37	5	0	0	0
	5 (F)				5	0	0	0

4 weeks. The aflatoxins and the observation periods were the same as for animals in experiment 1.

EXPERIMENT 3

This group of animals, after treatment with carbon tetrachloride in the same manner as described in experiment 1, was given eight weekly doses of the aflatoxin preparation and was observed for 37 weeks before necropsy studies.

EXPERIMENT 4

Ten rats were first given 5 per cent ethyl alcohol in water for 12 weeks. Four weeks after the discontinuation of alcohol, three weekly doses of the aflatoxins were given and the animals were sacrificed at the end of 12 weeks. The doses of the aflatoxins were the same as in the above experiments.

CONTROL GROUPS

Two groups of animals with no pretreatment were used as controls. One group of animals received three weekly doses of aflatoxins and was sacrificed on the 12th week; another group of animals, given eight consecutive weekly doses of aflatoxins, was maintained for 37 weeks until autopsy.

Complete autopsies were performed on all rats after sacrifice. Tissues were fixed in 10 per cent buffered formalin and processed routinely. Multiple tissue blocks from different parts of each liver were sampled and prepared for histologic examination. In addition to hematoxylin-eosin stain, Gomori reticulum, periodic acid-Schiff, and Masson trichrome stains were employed for liver sections.

RESULTS

Evaluation of the degree of cirrhotic change in livers caused by carbon tetrachloride and the development of

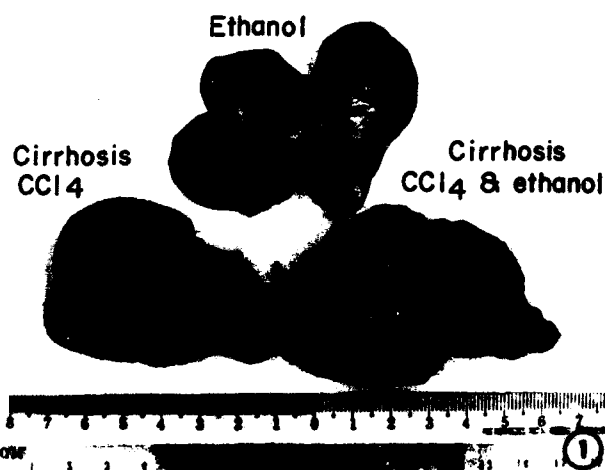


FIG. 1. Livers of rats used in the aflatoxin experiments and pretreated with ethanol, CCl_4 , or a combination of the two.

precancerous and cancerous lesions induced by aflatoxins is recorded in Table 1. Severe degrees of postnecrotic cirrhosis of a coarse nodular type were produced consistently by the combined administration of ethyl alcohol and CCl_4 (Fig. 1, lower right); however, mortality was high during this period. There was marked distortion of the hepatic architecture with abundant fibrous tissue. The hepatic cells in regenerative nodules of varying sizes disposed among the wide fibrous tissue septa had relatively uniform sized nuclei with fine, evenly distributed chromatin granules. In the groups of animals pretreated with CCl_4 alone, the gross picture of the liver showed a fine nodular cirrhosis of varying degrees. Interlobular fibrosis with increased regenerative activity of the liver cells was manifested by an increased number of binucleated hepatic cells; focal fatty changes were present. These were the characteristic histologic features of the livers examined before aflatoxins were administered. Mild to moder-

ate degrees of fatty change in the liver were present in the group of rats fed ethyl alcohol alone for 12 weeks.

In both short term experiments (experiments 1 and 2), about 50 per cent of animals with postnecrotic cirrhosis died either before the cessation of aflatoxin administration or during the period of observation. Even higher mortality was encountered in the group of cirrhotic animals given more aflatoxins and observed for longer periods of time. Thirteen of 20 animals died before the end of the experiment; of these, 11 were available for complete autopsies between 5 and 30 weeks following the first injection of the toxins. Hepatoma was found in three of the rats and parenchymal hepatic cell necrosis was commonly seen. These findings were in addition to nuclear atypism encountered in the liver cells. Severe pulmonary infection was invariably noted in animals that died early in the course of the experiment.

SHORT TERM EXPERIMENTS

After the administration of three doses of aflatoxins and after a 12-week observation period, the gross changes in rat livers varied from animal to animal. In some animals the liver retained the fine irregular nodularity of postnecrotic cirrhosis. Others exhibited large, protruding nodules of hepatic cell carcinoma (Fig. 2).

Seven of the 11 animals with postnecrotic cirrhosis of moderate degree and four of five animals having severe postnecrotic cirrhosis developed liver cancer evident on either gross or microscopic examination. The remaining animals in these two groups demonstrated marked atypical morphologic changes of hepatic cells. The atypical cells exhibited increased periodic acid-Schiff staining reactions and were characterized by eosinophilic cytoplasm, relatively large nuclei of variable size with heavy nuclear membranes, and acidophilic nucleoli (Fig. 3). Massive atypical hyperplasia of small sized liver cells was noted in the same instance (Fig. 3). Very often, the cells were arranged in radiating form around portal veins. The fully developed hepatoma was mostly of the trabecular type,

forming short cords or cylindrical masses closely resembling normal cord cells (Fig. 4). Occasional intravascular metastases were found in three animals (Fig. 5). Other animals demonstrated large hepatomas of acinar and alveolar types (Fig. 6). Ductal proliferation was conspicuous in the cirrhotic livers affected by the toxins in these groups.

In the group of animals fed 5 per cent ethanol, mild to moderate fatty changes of liver cells with no alteration of the lobular histologic architecture occurred. Characteristic Mallory bodies were present among the vacuolated hepatic cells. Following administration of aflatoxins for three weekly doses of 200 μ g. each with an observation period of 12 weeks, the liver cells as well as the vacuolated cytoplasm exhibited variable sized hyperchromatic nuclei. Elsewhere, foci of parenchymal necrosis were seen. No tumor growth was identified in the livers.

LONG TERM EXPERIMENT

After eight weekly doses of aflatoxins with a 37-week observation period, two of the seven animals developed hepatocarcinoma and three had advanced atypia of liver cells. In addition to the liver parenchymal changes, hyperplasia of atypical bile ductules in the proliferated fibrous tissue resembling cholangiocarcinoma was noted in two animals.

Apart from the hepatomas, an adenomatous type of carcinoma of the lung was demonstrated in one rat and benign adenomatous hyperplasia within lung tissues was found in eight animals. No metastasis secondary to liver cancer was present.

CONTROL GROUPS

Livers of control animals without pretreatment with either carbon tetrachloride or ethanol did not develop liver cancer following administration of aflatoxin. Enlarged nuclei of variable size in the hepatic cells, cytoplasmic vacuolation, and foci of parenchymal necrosis, however, were commonly seen.

DISCUSSION

Many studies of the histogenesis of liver cancer with chemical carcinogens have implicated hyperplastic nodules as a probable precancerous lesion.^{5, 10} Hepatoma caused by aflatoxin, however, has been adequately described but has not been associated with cirrhosis.^{7, 8} The influence of postnecrotic cirrhosis on aflatoxin carcinogenic effect appears evident from these experiments. In short term studies with low doses of toxins, it was observed that about 80 per cent of animals with postnecrotic liver cirrhosis developed hepatocarcinoma while, in animals without cirrhosis or with fatty metamorphosis alone, no tumor was found; only nuclear atypism of the hepatic cells occurred. The most striking observation was the demonstration of early transformation to cancer cells of the atypical regenerative liver cells within the cirrhotic nodules (Fig. 3). This may represent a pathway wherein the toxins affect the hyperplastic regenerative nodules of hepatic cells in postnecrotic cirrhosis. This may indicate that the somatic cell elements are the precursors of liver cell cancers. From what was observed, the rapidly proliferating



FIG. 2. Liver of a rat pretreated with CCl₄ followed by three injections of 200 μ g. of aflatoxins at 12 weeks. Note multiple large nodules of hepatoma, cicatricial contracture, and discrete small hyperplastic nodules on the surface (arrows).

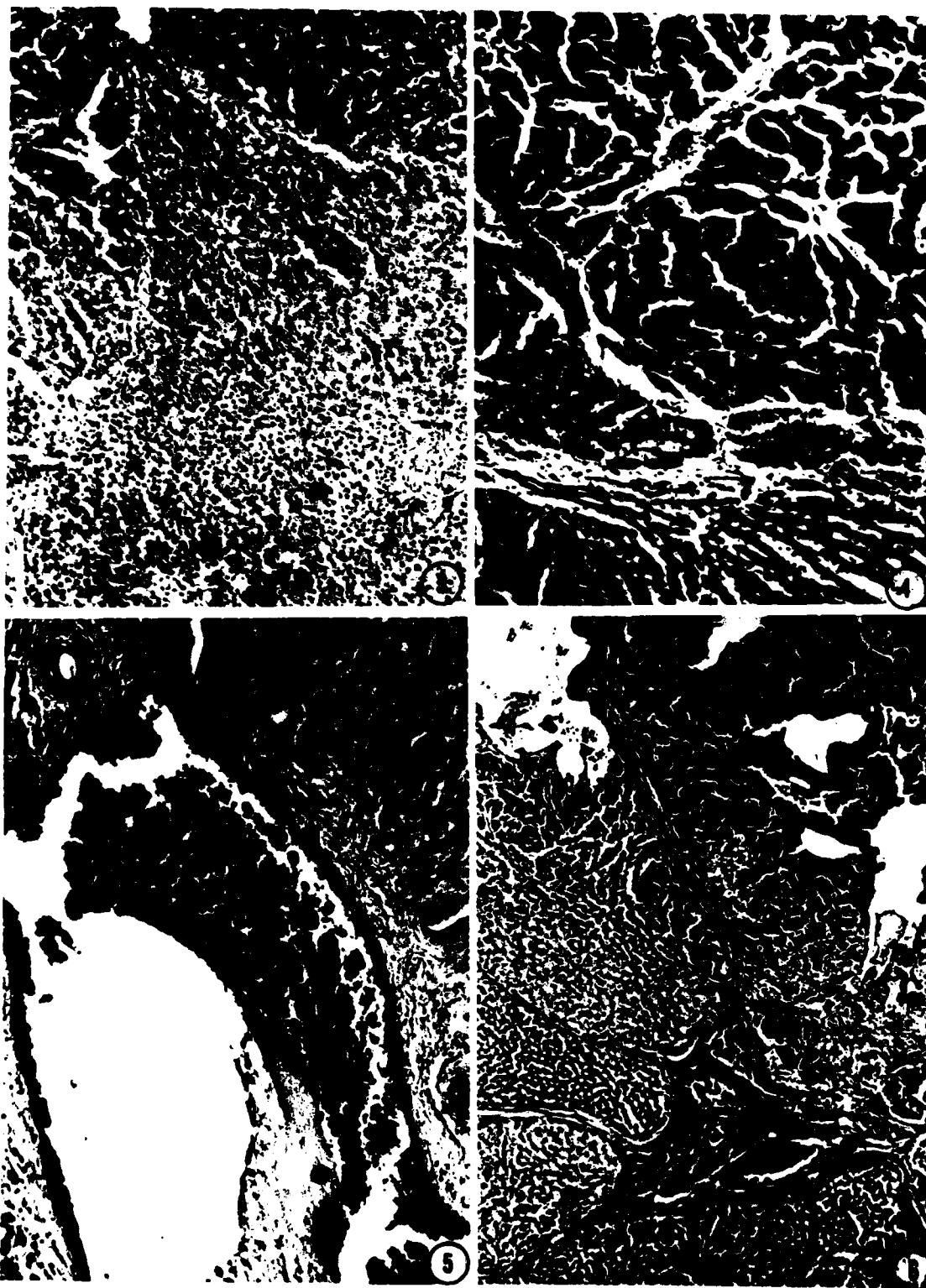


FIG. 3. Liver of a rat treated as in Figure 2 shows massive atypical hyperplasia of small sized liver cells. Note also the advanced nuclear atypism of liver cells adjacent to the small cell nodule. Cirrhosis was evidenced by perivascular fibrosis. $\times 120$.

FIG. 4. Liver of a rat shown in Figure 2. Note a histologically well differentiated, trabecular type hepatoma closely resembling normal cord cells. $\times 240$.

FIG. 5. Liver of a rat of the same group shows intravenous metastasis of well differentiated hepatic cancer cells. $\times 240$.

FIG. 6. Liver of a rat pretreated with CCl₄ and ethanol followed by three injections of 200 μ g. of aflatoxins at 12 weeks. A large nodule of acinar and alveolar type hepatoma was observed among the cirrhotic regenerative nodules. $\times 120$.

erating liver cells following CCl_4 administration developed rather primitive characteristics. It has already been shown that aflatoxin affects the biochemical action of cellular DNA.^{3, 13} Aflatoxin may possess a mutagenic mechanism as its carcinogenic agent which is more effective on primitive liver cells. Lowenstein and Lee⁶ have reported that aflatoxin affects hepatic cell division in cirrhosis induced by a choline-deficient diet. This study verifies the observation reported by Newberne, Harrington, and Wogan⁹ that suggested a potentiating effect of aflatoxin. A rather rapid development of liver cancer, however, was observed in this experimental model of postnecrotic type cirrhosis. Because of the small numbers of animals that survived at the end of the experiment, no attempt was made to correlate the severity of cirrhosis and sex differences with the development of hepatocarcinoma by the toxins.

Moreover, since the functions for the detoxification and degradation of aflatoxins are seemingly impaired in the cirrhotic liver, its toxicity might become enhanced and perhaps sustained for longer periods of time. The high mortality, especially in the long term experimental group, could be explained on this basis.

Aflatoxin is now known to be the most potent hepatocarcinogen discovered so far and is capable of causing hepatoma in male rats with a total dose of 400 μg . in 35 to 82 weeks.¹⁴ The doses employed in this experiment were comparatively low as far as aflatoxin B_1 was concerned, and the time required for genesis of cancer in the liver was greatly shortened. For any hepatotoxin that affects the liver, secondary factors may modify the histologic features of the liver parenchyma.¹¹ A direct intervening action of CCl_4 and/or ethanol on the influence of aflatoxin on liver seemed remote because of their metabolic clearance rates. Considerable time intervals of 4 to 6 weeks were arranged before the aflatoxin administration. Another secondary factor avoided was surgical biopsies in that these were considered to be an additional insult to the liver.

While it is a matter of fact that cirrhosis is intimately associated with primary liver cancer in man, the present data suggest that the regenerative liver cells in cirrhotic

nodules are more susceptible and sensitive to environmental carcinogens such as aflatoxins than normal hepatic cells.

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